Pro-inflammatory and anti-inflammatory antibodies against the heparin-binding protein (HBP)

Technical field of the invention

The present invention relates to providing antibodies against human heparin binding protein (hHBP) and using said antibodies for the manufacture of a medicament for treatment of Gram positive and/or Gram negative infections, sepsis, disseminated intravascular coagulation, modulation of inflammatory response, and/or prevention of cell apoptosis.

Background of the invention

A local infection or injury in any tissue rapidly attracts white blood cells into the affected region as part of the inflammatory response, which helps fight the infection or heal the wound. The inflammatory response is complex and is mediated by a variety of signalling molecules produced locally by different types of cells. Some of these molecules act on nearby capillaries, causing the endothelial cells to adhere less tightly to one another but making their surfaces adhesive to passing white blood cells. Other molecules act as chemoattractants for specific types of blood cells, such as monocytes, causing these cells to become polarised and crawl toward the source of the attractant.

White blood cells, specifically polymorphonuclear leukocytes (PMNs), produce a large variety of peptides involved in the inflammatory response. Among these peptides is the heparin-binding protein (HBP), which was first isolated from azurophile granules of human PMNs. A highly homologous peptide was also isolated from PMNs of porcine origin and has been named porcine heparin-binding protein (pHBP) (Flodgaard et al., 1991, Eur. J. Biochem. 197: 535-547; Pohl et al., 1990, FEBS Lett. 272: 200 ff.) HBP has otherwise been termed CAP37 (WO 91/00907, US 5,458,874 and 5,484,885) and azurocidin (Wilde et al. 1990, J. Biol. Chem. 265:2038-41).

Sequence analysis of HBP has revealed that the protein bears many similarities to serine proteases, which are important in inflammatory processes, e. g. neutrophil

elastase (47% homology) or protease 3 (43% homology), however HBP lacks protease activity due to mutations of two of three amino acids in the highly conserved catalytic triad. The structure of HBP appears from WO 89/08666 and Flodgaard et al., 1991 (Eur. J. Biochem. 197: 535-547).

HBP was originally studied because of its antibiotic and lipopolysaccharide binding properties (Gabay et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:5610-5614 and Pereira et al., 1993, Proc. Natl. Acad. Sci. USA 90: 4733-7). However, a number of experimental evidence now supports the concept that HBP is a multifunctional protein, and, in addition to its bactericidal role, is involved during the progression of inflammation due to its effect on the recruitment and activation of monocytes (Pereira et al., 1990, J. Clin. Invest. 85:1468-1476, and Rasmussen et al., 1996, FEBS Lett. 390:109-112), recruitment of T cells (Chertov et al., 1996, J. Biol. Chem. 271:2935-2940), as well as on the induced contraction of endothelial cells and fibroblasts (Ostergaard and Flodgaard, 1992, J. Leuk. Biol. 51:316-323). Ostergaard and Flodgaard (op. cit.) also disclose increased survival of monocytes treated with HBP. Furthermore, in animal models of fecal peritonitis, HBP treatment has been shown to rescue mice from an otherwise lethal injury (Mercer-Jones et al., 1996, In: Surgical Forum, pp. 105-108; Wickel et al., 1997, In: 4th International Congress on the Immune Consequences of Trauma, Chock and Sepsis, Munich, Germany, pp. 413-416).

It would be advantageous to develop new medicaments aimed to modulate the biological responses mediated by the HBP, in particular inflammatory response, using new agoinists or antagonists of the protein.

Summary of the invention

It was surprisingly found that monoclonal antibodies against human HBP (hHBP) are capable of modulating inflammatory response. Even more surprisingly, it was found that the antibodies produced by one clone are potent stimulators of the HBP-mediated inflammatory response, while the antibody produced by another clone do indeed reduce or even inhibit said response.

Accordingly, the present invention relates in one aspect to a pharmaceutical composition for the stimulating of at least one inflammatory response associated with hHBP or a homologue thereof, comprising an antibody against hHBP (SEQ ID NO: 1), said antibody capable of binding to an epitope within the sequence comprising amino acid residues 1 to 19 or 45 to 226 according to SEQ ID NO: 1 and thereby stimulating at least one inflammatory response mediated by hHBP.

Another aspect of the invention concerns a pharmaceutical composition for the inhibiting of at least one inflammatory response associated with human heparin binding protein (hHBP), or a homologue thereof, comprising an antibody against hHBP (SEQ ID NO: 1), said antibody capable of binding to an epitope within the sequence comprising amino acid residues 20 to 44 according to SEQ ID NO: 1 and thereby inhibiting at least one inflammatory response mediated by hHBP.

The invention discloses pro-inflammatory anti-hHBP monoclonal antibody produced by clone F19A5B1 (ECACC Ass. No.: 03090301) and anti-inflammatory anti-hHBP monoclonal antibody produced by clone F19A5B4 (ECACC Ass. No.: 03090302). The invention also relates to a method for producing the above antibodies and cells producing thereof.

Other aspects of the invention concern uses of the pharmaceutical compositions comprising anti-hHBP antibodies for the stimulation or inhibition of the inflammatory response. The invention also relates to uses antibodies F19A5B1 and F19A5B4 for (1) stimulating or inhibiting inflammatory response and/or (2) manufacture of a medicament for the treatment individuals having suppressed immune system, cancer, autoimmune diseases and/or trauma or individuals having a sustained inflammatory response.

The invention also relates to recombinant antibody molecules, antibody fragments, and other compounds, said molecules, fragments and compounds being capable of recognising the epitopes of the invention.

Detailed description of the invention

1. Inflammation

The present invention relates to providing antibodies and using said antibodies for the manufacture of a medicament for modulation of the inflammatory response.

Inflammation is a defence reaction caused by tissue damage due to a mechanical injury or bacterial, virus or other organism infection. The inflammatory response involves three major stages: first, dilation of capillaries to increase blood flow; second, microvascular structural changes and escape of plasma proteins from the bloodstream; and third, leukocyte transmigration through endothelium and accumulation at the site of injury and infection. The inflammatory response begins with a release of inflammatory mediators. Inflammatory mediators are soluble, diffusible molecules that act locally at the site of tissue damage and infection, and at more distant sites, influencing consequent events of the inflammatory response. Inflammatory mediators can be exogenous, e. g. bacterial products or toxins, or endogenous, which are produced within the immune system itself, as well as injured tissue cells, lymphocytes, mast cells and blood proteins.

In one aspect the present invention relates to an inflammatory response in connection with bacterial infection.

By "bacterial infection" in the present context is meant the invasion of normally sterile host tissue by bacteria. Bacterial infection of the invention may be due to invasion of either Gram negative or Gram positive bacteria, or a combination thereof or other infectious agents including fungi and virus. In one embodiment the present invention relates to the inflammatory response due invasion of Gram negative bacteria selected from the group comprising Acetobacteriaceae, Alcaligenaceae, Bacteroidaceae, Chromatiaceae, Enterobacteriaceae, Legionellaceae, Neisseriaceae, Nitrobacteriaceae, Pseudomonadaceae, Rhizobiaceae, Rickettsiaceae, Spirochaetaceae, Vibrionaceae, Brucella, Chromobacterium

In another embodiment the invention relates to an inflammatory response due to invasion by Gram positive bacteria selected from the group comprising Bacillaceae, Micrococcaceae (for example Staphylococcus aureus), Mycobacteriaceae (for example Staphylococcus pneumoniae), Peptococcaceae.

In an additional another embodiment the invention relates to an inflammatory response associated with sepsis, severe sepsis and/or septic shock.

By "sepsis" in the present context is meant the systematic inflammatory response to bacterial infection, characterised by one or more of the following conditions as a result of infection: temperature >38 °C or < 36 °C, heart rate > 90 beats/min, respiratory rate > 20 breaths/min or $PaCO_2$ < 32 torr (< 4.3 kPa), and WBC > 12 000 cells/mm^3 or < 4000 cells/ mm^3 or 10% immature (band) forms.

By "severe sepsis" in the present context is meant sepsis associated with organ dysfunction, hypoperfusion, or hypotension, hypoperfusion and hypotension abnormalities may include, but are not limited to, lactic acidosis (acidic condition in blood), oliguria (meaning reduction in urine production), or acute alteration in mental status.

By "septic shock" in the present context is meant sepsis with hypotension despite adequate fluid resuscitation, along with the presence of perfusion abnormalities that may include, but are not limited to, lactic acidosis, oliguria, or acute alteration in mental status.

In yet another embodiment the invention relates to an inflammatory response associated with disseminated intravascular coagulation (DIC).

By "DIC" in the present context is meant a pathophysiologic condition involving a continuum of events that occur in the coagulation pathway in association with a variety of well-defined clinical situations, including sepsis, major trauma, and abruptio placenta, and with laboratory evidence of the following: procoagulant activation, fibrinolytic activation, inhibitor consumption and biochemical evidence of end-organ damage or failure.

The present invention features pharmaceutical compositions comprising compounds capable of modulating at least one inflammatory response associated with the above pathological conditions.

In the present context by the term "inflammatory response" is meant a biological process or a series of biological reactions specifically associated with inflammation.

The invention concerns the inflammatory response due to biological activity of hHBP and selected from the group comprising

i) up-regulation of the gene expression in the immune cells, preferably monocytes/macrophages, leading to secretion of endogenous inflammatory mediators including receptors for inflammatory mediators and transcription factors involved in the signal tranduction of the inflammatory mediators, said mediators being preferably selected from the group comprising cytokines, selected from the group TNFalpha IL-1, IL-6, G-CSF, GM-CSF,M-CSF. Chemokines selected from the group comprising IL-8, MCP-1, receptors selected from the groupTissue factor and IL-2Ralpha,

ii) activation of the production of bradykinin by the phase contact system,

iii) increasing the blood concentration of monocytes and/or local accumulation thereof at the sites of inflammation,

iv) increasing the life-time of monocytes, neutrophils and other immune cells due to inhibition of apoptosis,

v) activation of expression of adhesion molecules by the vascular endothelial cells, said adhesion molecules being preferably selected from the group comprising PECAM, ICAM-1, E-selectins, VCAM-1,

vi) activation of the contact phase system producing bradykinin leading to an increased vascular permeability,

vii) increasing the phagocytic potential of monocytes/macrophages,

viii) up-regulation of class-II MHC.

By the term "modulating" of an inflammatory response is meant a capability of a compound to increase or decrease the threshold of the response. It is understood that the compound is capable of stimulating of inflammatory response when the threshold/upper limit of the response is increased in connection with using of the compound, and the compound is capable of inhibiting of inflammatory response when the threshold of the response is decreased.

In one aspect a pharmaceutical composition of the invention comprises a compound capable of stimulating at least one inflammatory response, a pro-inflammatory compound.

In another aspect the present invention relates to a pharmaceutical composition comprising a compound capable of inhibiting at least one inflammatory response, an anti-inflammatory compound.

The present invention in a preferred embodiment concerns at least one of the above inflammatory responses mediated by hHBP.

Pro-inflammatory compound

In one aspect the invention relates to a pro-inflammatory compound being an antibody against hHBP.

The pro-inflammatory antibody of the invention is an antibody, which is

i) capable to recognise an epitope located within a sequence comprising amino acid residues 1 to 19 or 45 to 225 according to the sequence identified in SEQ ID NO:1;

ii) capable of stimulating the inflammatory response associated with one or more biological activities of hHBP or a hHBP fragment comprising amino acids 20-44 according to the sequence identified as SEQ ID NO: 1,

i) capable of stimulating at least one of the above described inflammatory responses, wherein the antibody are often capable of stimulating any two or more of the above responses.

A pro-inflammatory antibody of the invention may in one embodiment be capable of stimulating at least one of the above described inflammatory responses in synergistic action with bacterial products including, but not limited to LPS (Lipopolysaccharide), PGN (peptidoglycan), LTA (Lipotechoic acid), MDP (muramyldipeptide) and PCW (purified cell wall from bacteria) present in the blood or in a biological sample collected from an individual. In another embodiment, a pro-inflammatory antibody of the invention is capable of stimulating at least one of the above described inflammatory responses in the absence of the above mentioned bacterial products.

The pro-inflammatory antibody may be either polyclonal or monoclonal. In a preferred embodiment the pro-inflammatory antibody is a monoclonal antibody

against hHBP. A preferred pro-inflammatory antibody of the invention is the antibody produced by a cell of clone F17A5B1 (ECACC Ass. No.: 03090301)

Another preferred pro-inflammatory antibody of the invention is the antibody that is capable of stimulating the production of cytokine IL-6.

In another aspect the invention relate to a fragment of the above antibody, said fragment being capable of the features of a pro-inflammatory antibody of above, such as a capability of binding to an epitope of hHBP or a hHBP homologue, said epitope being specifically recognisable by said pro-inflammatory antibody, and stimulating at least one of the inflammatory responses describes above.

Still, in another aspect the invention concerns a recombinant protein, said recombinant protein comprising a fragment of the above pro-inflammatory antibody, or a full-length antibody molecule of above, and which is capable of activity of said fragment or said antibody molecule.

An antibody, which is capable of binding to the epitope of hHBP specifically recognisable by the above pro-inflammatory antibody, by a fragment thereof or a recombinant protein thereof, is also in the scope of the invention as a pro-inflammatory compound capable of stimulating at least one of the above described inflammatory responses. It is understood that the latter antibody is an antibody against another antigen then hHBP, or hHBP homologue (e. g. pHBP or hNEL), said antibody, however, are capable of binding to an epitope of the invention and stimulating inflammatory response.

Anti-inflammatory compound

It is another important objective of the invention to provide a new anti-inflammatory compound, which is capable of serving as an inhibitor of the sustained inflammatory response.

The continuous presence of inflammatory mediators, such as for example TNF alpha in the body in response to sustained presence of bacterial products or even live bacteria locally during days or weeks following trauma and/or infection promotes the reactions to inflammation, such as, for example, heat, swelling, and pain. The sus-

tained inflammatory response has been proven to be very harmful to the body. If the bacterial products or live bacteria become spread universally in the body from their local focus the inflammatory reaction becomes overwhelming and out of control and leads to sepsis which eventually progress further to severe sepsis and septic shock. Anti-inflammatory peptides may be used to block or suppress the overwhelming sustained inflammatory response represented by a massive and harmful cytokine cascade in the blood and vital organs such as lung, liver intestine, brain and kidneys.

In the present context by the term "anti-inflammatory compound" is meant a compound which is capable of

i) decreasing or inhibiting the gene expression in the immune cells, preferably monocytes/macrophages in response to bacterial products, live bacteria or trauma to produce endogenous inflammatory mediators including receptors for inflammatory mediators and transcription factors involved in the signal transduction of the inflammatory mediators, said mediators being preferably selected from the group comprising cytokines, selected from the group TNFalpha IL-1, IL-6, G-CSF, GM-CSF, M-CSF, chemokines selected from the group comprising IL-8, MCP-1, or receptors selected from the group Tissue factor and IL-2Ralpha. and/or

ii) decreasing or inhibiting the production bradykinin by the phase contact system, and/or;

iii) decreasing or inhibiting the chemoattraction of monocytes to the sites of inflammation, and/or

iv) decreasing or inhibiting the expression of the adhesion molecules by vascular endothelial cells, said adhesion molecules being preferably selected from the group comprising PECAM, ICAM-1, E-selectins, VCAM-1 and/or

v) decreasing or inhibiting the activation of the contact phase system to produce bradykinin leading to increased vascular permeability, and/or

vi) stimulating the synthesis of an anti-inflammatory mediator selected from the group of IL-10 and IL-12, and/or

vii) decreasing the concentration of endotoxin in body fluids of septic patients, and/or

The present invention relates in one aspect to the anti-inflammatory compound being an antibody against hHBP, or a hHBP homologue, such as for example porcine HBP (pHBP) or human neutrophil elastase (hNEL),

i) capable of recognising an epitope located within a sequence comprising amino acid residues 20 to 44 according to the sequence identified in SEQ ID NO:1;

ii) capable of inhibiting the inflammatory response associated with one or more biological activities of hHBP or a hHBP fragment comprising amino acids 20-44 according to the sequence identified as SEQ ID NO: 1,

iii) capable of at least one of the above activities of an anti-inflammatory compound, more preferable at least two of the above activities, even more preferable at least tree of the above activities, even more preferable at least four of the above activities, even more preferable at least five of the above activities, even more preferable at least six of the above activities, even more preferable at least seven of the below activities, and most preferably eight of the above activities.

In a preferred embodiment the invention relates to an anti-inflammatory antibody capable of inhibiting the secretion of cytokine IL-6 from monocytes in response to bacterial products including, but not limited to, LPS (Lipopolysaccharide), PGN (peptidoglycan), LTA (Lipotechoic acid), MDP (muramyldipeptide) and PCW (purified cell wall from bacteria). In another preferred embodiment the invention relates to an anti-inflammatory antibody capable of decreasing or inhibiting the secretion of cytokine IL-6 from monocytes activated by HBP, a HBP fragment comprising amino acid residues 20-44 in the absence of the mentioned bacterial products, and/or in the absence of other monocyte activating factors and/or conditions such as for example conditions of trauma, auto-immune disease, cancer, sepsis, severe sepsis, septic shock and/or disseminated intravascular coagulation.

The anti-inflammatory antibody may be either a polyclonal or monoclonal antibody. In a preferred embodiment the anti-inflammatory antibody of the invention is a monoclonal antibody against hHBP. A preferred monoclonal antibody of the invention is the antibody produced by a cell of clone F17A5B4 (ECACC Ass. No.: 03090302)

In another aspect the invention relates to a fragment of the above anti-inflammatory antibody, said fragment being capable of the features of the anti-inflammatory antibodies of above, such as a capability of binding to an epitope of hHBP or a hHBP homologue, and thereby inhibit at least one of the inflammatory responses describes above, said epitope being specifically recognisable by the anti-inflammatory antibody of above,.

Still in another aspect the invention concerns a recombinant protein, which comprises a fragment of the above anti-inflammatory antibody, or the full-length anti-inflammatory antibody molecule of above, and which is capable of activity of said fragment or antibody molecule.

Antibody molecules specific for other antigens then hHBP, or the hHBP homologues of the invention, which, however, are capable of binding to the epitope of hHBP, which specifically recognised by the above anti-inflammatory antibody, fragment thereof or recombinant protein comprising thereof, are also in the scope of the invention as anti-inflammatory compounds capable of inhibiting at least one of the inflammatory responses mediated by hHBP.

2. Antibody

It is an objective of the present invention to provide an antibody capable of selectively binding to hHBP (SEQ ID NO: 1), a fragment thereof, recombinant protein thereof or homologue thereof, such as for example pHBP (SEQ ID NO: 588) or hNEL (SEQ ID NO: 589).

Antibody molecules belong to a family of plasma proteins called immunoglobulins, whose basic building block, the immunoglobulin fold or domain, is used in various forms in many molecules of the immune system and other biological recognition systems. A typical immunoglobulin has four polypeptide chains, containing an antigen binding region known as a variable region and a non-varying region known as the constant region.

Native antibodies and immunoglobulins are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical

heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (VH) followed by a number of constant domains. Each light chain has a variable domain at one end (VL) and a constant domain at its other end. The constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains (Clothia et al., J. Mol. Biol. 186, 651-66, 1985); Novotny and Haber, Proc. Natl. Acad. Sci. USA 82, 4592-4596 (1985).

Depending on the amino acid sequences of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are at least five (5) major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g. IgG-1, IgG-2, IgG-3 and IgG-4; IgA-1 and IgA-2. The heavy chains constant domains that correspond to the different classes of immunoglobulins are called alpha (α), delta (δ), epsilon (ε), gamma (γ) and mu (μ), respectively. The light chains of antibodies can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino sequences of their constant domain. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "variable" in the context of variable domain of antibodies, refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies. The variable domains are for binding and determine the specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed through the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) also known as hypervariable regions both in the light chain and the heavy chain variable domains.

The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely a adopting a β-sheet configuration, connected by three CDRs, which

form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies. The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

An antibody that is contemplated for use in the present invention thus can be in any of a variety of forms, including a whole immunoglobulin, an antibody fragment such as Fv, Fab, and similar fragments, a single chain antibody which includes the variable domain complementarity determining regions (CDR), and the like forms, all of which fall under the broad term "antibody", as used herein. The present invention contemplates the use of any specificity of an antibody, polyclonal or monoclonal, and is not limited to antibodies that recognize and immunoreact with a specific antigen. In preferred embodiments, in the context of both the therapeutic and screening methods described below, an antibody or fragment thereof is used that is immunospecific for an antigen or epitope of the invention.

The term "antibody fragment" refers to a portion of a full-length antibody, generally the antigen binding or variable region. Examples of antibody fragments include Fab, Fab', $F(ab')_2$ and Fv fragments. Papain digestion of antibodies produces two identical antigen binding fragments, called the Fab fragment, each with a single antigen binding site, and a residual "Fc" fragment, so-called for its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen binding fragments that are capable of cross-linking antigen, and a residual other fragment (which is termed pFc'). Additional fragments can include diabodies, linear antibodies, single-chain antibody molecules, and multispecific antibodies formed from antibody fragments. As used herein, "functional fragment" with respect to antibodies, refers to Fv, F(ab) and $F(ab')_2$ fragments.

Antibody fragments may be as small as about 4 amino acids, 5 amino acids, 6 amino acids, 7 amino acids, 9 amino acids, about 12 amino acids, about 15 amino acids, about 17 amino acids, about 18 amino acids, about 20 amino acids, about 25 amino acids, about 30 amino acids or more. In general, an antibody fragment of the invention can have any upper size limit so long as it is has similar or immunological

properties relative to antibody that binds with specificity to an epitope located within a sequence selected from the group consisting of SEQ ID NO: 1, 588 and 589, such as an epitope comprising at least one of the sequences identified in SEQ ID NO: 2-587, for example such as an epitope located within a sequence comprising amino acid residues 1 to 19 or 45 to 225 according to the sequence identified in SEQ ID NO:1.

Antibody fragments retain some ability to selectively bind with its antigen or receptor. Some types of antibody fragments are defined as follows:

(1) Fab is the fragment that contains a monovalent antigen-binding fragment of an antibody molecule. A Fab fragment can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain.

(2) Fab' is the fragment of an antibody molecule can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain. Two Fab' fragments are obtained per antibody molecule.

Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region.

(3) $(Fab')_2$ is the fragment of an antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction.

(4) $F(ab')_2$ is a dimer of two Fab' fragments held together by two disulfide bonds.

Fv is the minimum antibody fragment that contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in a tight, non-covalent association (V_H -V_L dimer). It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the V_H -V_L dimer. Collectively, the six CDRs confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

(5) Single chain antibody ("SCA"), defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy

chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule. Such single chain antibodies are also referred to as "single-chain Fv" or "sFv" antibody fragments. Generally, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains that enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in The Pharmacology of Monoclonal Antibodies 113: 269-315 Rosenburg and Moore eds. Springer-Verlag, NY, 1994.

The term "diabodies" refers to a small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161, and Hollinger et al., Proc. Natl. Acad Sci. USA 90: 6444-6448 (1993).

The preparation of polyclonal antibodies is well-known to those skilled in the art. See, for example, Green et al. 1992. Production of Polyclonal Antisera, in: Immunochemical Protocols (Manson, ed.), pages 1-5 (Humana Press); Coligan, et al., Production of Polyclonal Antisera in Rabbits, Rats Mice and Hamsters, in: Current Protocols in Immunology, section 2.4.1, which are hereby incorporated by reference.

The preparation of monoclonal antibodies likewise is conventional. See, for example, Kohler & Milstein, Nature, 256:495 (1975); Coligan, et al., sections 2.5.1-2.6.7; and Harlow, et al., in: Antibodies: A Laboratory Manual, page 726 (Cold Spring Harbor Pub. (1988)), which are hereby incorporated by reference. Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. See, e.g., Coligan, et al., sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3; Barnes, et al., Purification of Immunoglobulin G (IgG). In: Methods in Molecular Biology, 1992, 10:79-104, Humana Press, NY.().

Methods of *in vitro* and *in vivo* manipulation of monoclonal antibodies are well known to those skilled in the art. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature 256, 495 (1975), or may be made by recombinant methods, e.g., as described in US 4,816,567. The monoclonal antibodies for use with the present invention may also be isolated from phage antibody libraries using the techniques described in Clackson et al., 1991, Nature 352: 624-628, as well as in Marks et al., 1991, J Mol Biol 222: 581-597. Another method involves humanizing a monoclonal antibody by recombinant means to generate antibodies containing human specific and recognizable sequences. See, for review, Holmes, et al., 1997, J Immunol 158:2192-2201 and Vaswani, et al., 1998, Annals Allergy, Asthma & Immunol 81:105-115.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In additional to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired

biological activity (US 4,816,567); Morrison et al., 1984, Proc Natl Acad Sci 81, 6851-6855.

Methods of making antibody fragments are also known in the art (see for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, NY, 1988, incorporated herein by reference). Antibody fragments of the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, in US 4,036,945 and US 4,331,647, and references contained therein. These patents are hereby incorporated in their entireties by reference.

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody. For example, Fv fragments comprise an association of V_H and V_L chains. This association may be noncovalent or the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise V_H and V_L chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow, et al., 1991, In: Methods: A Companion to Methods in Enzymology, 2:97; Bird et al., 1988, Science 242:423-426; US 4,946,778; and Pack, et al., 1993, BioTechnology 11:1271-77.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") are often involved in antigen recognition and binding. CDR peptides can be obtained by cloning or constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick, et al., Methods: a Companion to Methods in Enzymology, Vol. 2, page 106 (1991).

The invention contemplates human and humanized forms of non-human (e.g. murine) antibodies. Such humanized antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a nonhuman species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity.

In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, humanized antibodies will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see: Jones et al., 1986, Nature 321, 522-525; Reichmann et al., 1988, Nature 332, 323-329; Presta, 1992, Curr Op Struct Biol 2:593-596; Holmes et al., 1997, J Immunol 158:2192-2201 and Vaswani, et al., 1998, Annals Allergy, Asthma & Immunol 81:105-115.

The invention provides both polyclonal and monoclonal antibodies. The generation of antibodies may be achieved by standard methods of the art for producing polyclonal and monoclonal antibodies using a natural or recombinant HBP polypeptide or fragment thereof as an antigen. Such antibodies would be in a preferred embodiment generated using a naturally occurring or recombinantly produced hHBP, or hHBP-related polypeptides, namely pHBP and hNEL, having amino acid sequences set forth in SEQ ID NO: 1, 588 or 589 correspondingly, or variants or fragments thereof, or, in a more preferred embodiment, using fragments of said polypeptides, wherein said fragments would meet at least two of the following criteria:

(i) being a natural or synthetic contiguous amino acid sequence of at least 8 amino acids derived from any of the sequences identified as SEQ ID NOS: 1, 588 or 589;

(ii) being a natural or synthetic contiguous amino acid sequence of at least 8 amino acids comprising at least one amino acid sequence selected from any of the sequences identified as SEQ ID NOS: 2-587.

The antibodies may also be produced *in vivo* by the individual to be treated, for example, by administering an immunogenic fragment according to the invention to said individual. Accordingly, the present invention further relates to a vaccine comprising an immunogenic fragment described above.

In one preferred embodiment the invention relates to an antibody, or antibody fragment, capable of binding to an epitope comprising a fragment of the amino acid sequence set forth SEQ ID NO: 1, said fragment consisting of the amino acid residues 1 to 19 or 45 to 225 according to SEQ ID NO: 1. Preferably that the epitope is located within the sequence hHBP (SEQ ID NO: 1).

In another preferred embodiment the invention relates to an antibody, capable of binding to an epitope comprising a fragment of the amino acid sequence set forth SEQ ID NO: 1, said fragment consisting of the amino acid residues 20 to 44 according to SEQ ID NO: 1. Preferably that the epitope is located within the sequence of hHBP (SEQ ID NO: 1).

It is understood that the latter antibodies are raised against hHBP, a fragment of hHBP or hHBP homologue. In particular, the invention concerns a monoclonal antibody against hHBP. In one preferred embodiment the monoclonal antibody is antibody F19A5B1. According to the invention antibody F19A5B1 is capable of binding to an epitope comprising one or more amino acid residues of the sequence consisting of amino acid residues 1 to 19 or 45 to 225 of the sequence set forth in SEQ ID NO: 1, said antibody is capable of stimulating at least one inflammatory response associated with one or more biological activities of hHBP or a hHBP fragment comprising amino acids 20-44 according to the sequence identified as SEQ ID NO: 1. In another preferred embodiment the monoclonal antibodies is antibody F19A5B4. According to the invention antibody F19A5B4 is capable of binding to an epitope comprising one or more amino acid residues of the sequence consisting of amino acid residues 20 to 44 of the sequence set forth in SEQ ID NO: 1, said antibody is capable of inhibiting at least one inflammatory response associated with one or more biological activities of hHBP or a hHBP fragment comprising amino acids 20-44 according to the sequence identified as SEQ ID NO: 1.

In still another embodiment, the invention relates to an antibody capable of recognising an epitope of the above embodiments, wherein said antibody is not anti-hHBP antibody. An example of such antibody may be an antibody raised against recombinant or natural protein or peptide, said protein or peptide comprising an amino acid sequence comprising the epitope(s) described above.

In another aspect, the application relates to a method for producing an antibody of the invention, said method comprising a step of providing hHBP or an immunogenic fragment of hHBP, or related immunogenic peptides/polypeptides described above, and using the provided immunogenes for the production of an antibody capable of modulating of inflammatory response by any of the methods described above.

Production of immunogenic peptide sequences and/or antibody fragments

The immunogenic molecules comprising hHBP, pHBP, hNLA and fragments thereof, of the present invention, or antibody and fragments thereof may be prepared by conventional synthetic methods, recombinant DNA technologies, enzymatic cleavage of full-length proteins which the peptide sequences are derived from, or a combination of said methods.

1. Synthetic preparation

The methods for synthetic production of peptides are well known in the art. Detailed descriptions as well as practical advice for producing synthetic peptides may be found in Synthetic Peptides: A User's Guide (Advances in Molecular Biology), Grant G. A. ed., Oxford University Press, 2002, or in: Pharmaceutical Formulation: Development of Peptides and Proteins, Frokjaer and Hovgaard eds., Taylor and Francis, 1999.

Peptides may for example be synthesised by using Fmoc chemistry and with Acm-protected cysteins. After purification by reversed phase HPLC, peptides may be further processed to obtain for example cyclic or C- or N-terminal modified isoforms. The methods for cyclization and terminal modification are well-known in the art and described in detail in the above-cited manuals.

In a preferred embodiment, immunogenic peptide sequences of the invention are produced synthetically, in particular, by the Sequence Assisted Peptide Synthesis (SAPS) method.

2. Recombinant production

The DNA sequence encoding a immunogenic peptide or full-length protein of the invention, or an antibody molecule of the invention may be prepared synthetically by established standard methods, e.g. the phosphoamidine method described by Beaucage and Caruthers, 1981, Tetrahedron Lett. 22:1859-1869, or the method described by Matthes et al., 1984, EMBO J. 3:801-805. According to the phosphoamidine method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

The DNA sequence encoding a peptide may also be prepared by fragmentation of the DNA sequences encoding the corresponding full-length protein of peptide origin, using DNAase I according to a standard protocol (Sambrook et al., Molecular cloning: A Laboratory manual. 2 rd ed., CSHL Press, Cold Spring Harbor, NY, 1989). The present invention relates to full-length proteins selected from the groups of proteins identified above. The DNA encoding the full-length proteins of the invention may alternatively be fragmented using specific restriction endonucleases. The fragments of DNA are further purified using standard procedures described in Sambrook et al.,

Molecular cloning: A Laboratory manual. 2 rd ed., CSHL Press, Cold Spring Harbor, NY, 1989.

The DNA sequence encoding a full-length protein may also be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the full-length protein by hybridisation using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989). The DNA sequence may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202 or Saiki et al., 1988, Science 239:487-491.

The DNA sequence is then inserted into a recombinant expression vector, which may be any vector, which may conveniently be subjected to recombinant DNA procedures. The choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding a peptide or a full-length protein should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence, which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the coding DNA sequence in mammalian cells are the SV 40 promoter (Subramani et al., 1981, Mol. Cell Biol. 1:854-864), the MT-1 (metallothionein gene) promoter (Palmiter et al., 1983, Science 222: 809-814) or the adenovirus 2 major late promoter. A suitable promoter for use in insect cells is the polyhedrin promoter (Vasuvedan et al., 1992, FEBS Lett. 311:7-11). Suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., 1980, J. Biol. Chem. 255:12073-12080; Alber and Kawasaki, 1982, J. Mol. Appl. Gen. 1: 419-434) or alcohol dehydrogenase genes (Young et al., 1982, in Genetic Engineering of Microorganisms for Chemicals, Hollaender et al, eds., Plenum Press, New York), or

the TPI1 (US 4,599,311) or ADH2-4c (Russell et al., 1983, Nature 304:652-654) promoters. Suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., 1985, EMBO J. 4:2093-2099) or the tpiA promoter.

The coding DNA sequence may also be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) promoters. The vector may further comprise elements such as polyadenylation signals (e.g. from SV 40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV 40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The recombinant expression vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV 40 origin of replication. The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or one which confers resistance to a drug, e.g. neomycin, hydromycin or methotrexate.

The procedures used to ligate the DNA sequences coding the peptides or full-length proteins, the promoter and the terminator, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., op.cit.).

To obtain recombinant peptides of the invention the coding DNA sequences may be usefully fused with a second peptide coding sequence and a protease cleavage site coding sequence, giving a DNA construct encoding the fusion protein, wherein the protease cleavage site coding sequence positioned between the HBP fragment and second peptide coding DNA, inserted into a recombinant expression vector, and expressed in recombinant host cells. In one embodiment, said second peptide selected from, but not limited by the group comprising glutathion-S-reductase, calf thymosin, bacterial thioredoxin or human ubiquitin natural or synthetic variants, or peptides thereof. In another embodiment, a peptide sequence comprising a prote-

ase cleavage site may be the Factor Xa, with the amino acid sequence *IEGR*, enterokinase, with the amino acid sequence *DDDDK*, thrombin, with the amino acid sequence *LVPR/GS*, or *Acharombacter lyticus,* with the amino acid sequence X*K*X, cleavage site.

The host cell into which the expression vector is introduced may be any cell which is capable of expression of the peptides or full-length proteins, and is preferably a eukaryotic cell, such as invertebrate (insect) cells or vertebrate cells, e.g. *Xenopus laevis* oocytes or mammalian cells, in particular insect and mammalian cells. Examples of suitable mammalian cell lines are the HEK293 (ATCC CRL-1573), COS (ATCC CRL-1650), BHK (ATCC CRL-1632, ATCC CCL-10) or CHO (ATCC CCL-61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159, 1982, pp. 601-621; Southern and Berg, 1982, J. Mol. Appl. Genet. 1:327-341; Loyter et al., 1982, Proc. Natl. Acad. Sci. USA 79: 422-426; Wigler et al., 1978, Cell 14:725; Corsaro and Pearson, 1981, in Somatic Cell Genetics 7, p. 603; Graham and van der Eb, 1973, Virol. 52:456; and Neumann et al., 1982, EMBO J. 1:841-845.

Alternatively, fungal cells (including yeast cells) may be used as host cells. Examples of suitable yeast cells include cells of *Saccharomyces spp.* or *Schizosaccharomyces spp.*, in particular strains of *Saccharomyces cerevisiae*. Examples of other fungal cells are cells of filamentous fungi, e.g. *Aspergillus spp.* or *Neurospora spp.*, in particular strains of *Aspergillus oryzae* or *Aspergillus niger*. The use of *Aspergillus spp.* for the expression of proteins is described in, e.g., EP 238 023.

The medium used to culture the cells may be any conventional medium suitable for growing mammalian cells, such as a serum-containing or serum-free medium containing appropriate supplements, or a suitable medium for growing insect, yeast or fungal cells. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection).

The peptides or full-length proteins recombinantly produced by the cells may then be recovered from the culture medium by conventional procedures including sepa-

rating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. HPLC, ion exchange chromatography, affinity chromatography, or the like.

3. Screening assays

Antibodies provided by any of the above described methods are preferably aimed for the manufacture of a medicament for the treatment of a pathological condition, wherein inhibiting or stimulating of inflammatory response is required. Accordingly, produced antibodies are further screened for their biological activity to be selected as useful for the purpose of the invention.

In the present content by "biological activity" is meant that a capability to demonstrate at least one of the following biological activities: (1) stimulating/inhibiting the production of IL-1, IL-6, IL-8, GCSF, GM-CSF, M-CSF, TNF-α, MCP-1, group Tissue factor, IL-2R-α; (2) enhancing/decreasing the bactericidal, chemotactic and/or anti-apoptotic activity of hHBP, (3) stimulating/inhibiting the vascular permeability, expression of adhesion molecules PECAM or ICAM1 by endothelial cells, and/or production of bradykinin dependent on hHBP; (4) stimulating/inhibiting the phagocytic potential of the cells, and/or (5) up/down-regulating class-II MHC.

In one preferred embodiment an antibody of the invention is able to demonstrate at least one of the above activities. In another embodiment, an antibody is capable of two or more activities of above.

Methods for evaluating of the above listed biological activities of the antibodies according to the invention are well known in art, and a number of assays for evaluating the biological activity of the antibody are available at the present.

One of such assays useful for the evaluation of chemotactic activity may for example be a method according to Cates et al. (in Leukocyte chemotaxis, p 67. Gallin and Quie eds, Raven Press, NY, 1978), or Keire et al. (J. Biol. Chem. 2001, 276: 48847-53).

To evaluate the bactericidal activity of a medicament comprising the antibody the assay described by Shafer et al. (Infect. Immun. 1986, 53:651-55) may be used.

The measuring of cell apoptopsis in the presence or in the absence of the present antibodies may for example be done according to Linde et al. (Anal. Biochem. 2000, 280:186-8).

The vascular permeability may be determined by using the assay as described by Gautam et al. in 1998 (Br J Pharmacol 1998 Nov;125(5):1109-14)

The changes in expression of different polypeptides, such as for example IL-1, IL-6, IL-8, TNF-α, thrombospondin, PECAM or ICAM in the presence of the antibodies according to the invention may, for example, be evaluated either by reverse phase transcriptase, immunoassay, immunoblotting, or immunostaining of the treated cells grown in culture. Secretion of the mediators of inflammation, such as for example IL-1, IL-6, IL-8, TNF-α, may be evaluated in Whole blood screening assay (WB) described below.

Human whole blood (WB) samples contain besides red cells, platelets and plasma the white blood cells including the neutrophils and monocytes. Neutrophils and monocytes have receptors for bacterial products such as LPS, PGN and LTA. The bacterial products react directly or via specific binding proteins to receptors on the monocytes thereby stimulating them to secrete and release inflammatory cytokines such as IL-1, IL-6, and TNF-α. HBP and HBP fragments, specifically the fragments comprising 20-44 aa according to SEQ ID NO: 1 or 2 do not influence the production of cytokines when they present in the blood, however they can significantly amplify cytokine synthesis and secretion induced by bacterial products. In the assay the amplification of 160 µM HBP per ml WB typically leads to at least three-fold amplification of the cytokine secretion.

In the screening assay LPS from the E. coli may be used to stimulate the monocytes in WB (anti-coagulated by use of citrate) to secrete IL-6 and activity of the antibodies may be evaluated in relation to the amount of IL-6 in plasma subsequently separated from WB. The activity may be related to the capability to:

1. increase IL-6 secretion in absence of bacterial products

2. increase IL-6 secretion in presence of bacterial products
3. decrease IL-6 secretion in presence of bacterial products
4. inhibit amplification of IL-6 secretion induced by intact HBP

All operations must be carried out in LAF cabinet by observance of stringent aseptic techniques. All test tubes, pipette tips etc. must be pyrogen-free. Buffers must be prepared by use of sterile, pyrogen water, preferably water for injection. Usage of 0.1 % pyrogen-free BSA/PBS for all dilutions is recommended.

The assay may be performed as the following:

- Add 20 µl of the antibody (in concentrations from 25 to 2500 µg/ml) to 100 µl freshly drawn (less than 4 hours old) citrate whole blood from a healthy human volunteer.
- Add 20 µl bacterial component (LPS, LTA or PGN) in concentrations from 5 to 5000 ng/ml, preferably 50 to 500 ng/ml.
- Mix well and incubate for 16-18 hours in an atmosphere of 5 % carbon dioxide and at least 95 % relative humidity.
- At the end of the incubation add at least 5 volumes (700 µl) 0.1 % BSA/PBS and mix well.
- Centrifuge 10 min. at 10.000 g.
- Aspirate 500 µl supernatant.
- Determine the level of IL-6 by a specific human immune assay for human IL-6 with sensitivity of at least 3 pg/ml, e.g. Human IL-6 Kit from RnD Systems (cat. no. D 6050).

Negative controls may be 100 µl WB plus 40 µl 0.1 % BSA/PBS.
Positive control may be 100 µl WB plus 20 µl LPS (same concentration as used for testing the peptide) and 20 µl 0.1 % BSA/PBS.

5. Functional antibody

A number of experimental evidence now supports the concept that HBP is a multi-functional protein, and, in addition to its bactericidal role, is involved during the progression of inflammation due to its effect on the recruitment and activation of monocytes, recruitment of T cells, as well as on the induced contraction of endothelial cells and fibroblasts.

There has been no identification of a HBP receptor, but receptor-like structures or binding sites of HBP have been identified. HBP is a dipole separated by a hydrophobic cleft and it is therefore capable to interact with both positively and negatively charge surfaces and molecules and with hydrophobic molecules and epitomes. The charged surface areas (the epitomes) of HBP are important for several of its functions. Without being bound by theory some of such functions are described below:

It has been demonstrated that HBP's positively charged epitomes bind to negatively charged macromolecules such as the heparan sulphate and chondroitin sulphate side chains of the proteoglycans, which are present at the surface of nearly every adherent mammalian cells. Proteoglycans are proteins with long carbohydrate chains of the glucosaminoglycans (GAG) type attached. They have recently been recognized as an important part of the signaling mechanism between cells. The proteoglycans are today recognized as co-receptors that can influence how e.g. the growth factor interacts with its receptor. Co-receptors affect which signal molecules bind to the receptor, how strong the interaction is or how far the signal spreads. Co-receptors regulate such decisions as when the cell divides, what type of proteins it manufactures and even if it should die. HBP has been shown to bind to the carbohydrate part (e.g. heparan sulphate) of the syndecan family of proteoglycans, which play an important role in internalization of proteins. The binding of HBP to such proteoglycans lead to uptake of HBP into endothelial cells and probably other cell types as well. Heparan sulphate and similar highly charged negative molecules of the glucosaminoglycan type may therefore serve as binding sites for HBP, mediating many of its diverse regulatory functions. In this context it is should be noted that heparan sulphate and similar glucosaminoglycans are not just simple negatively charged molecules mediating a non-specific ionic interaction. In contrast e.g. the heparan sulphate are synthesized such that very diverse and subtle variations in the structure are achieved. Accordingly, the synthesized heparan sulphate molecules may fit only very specific positively charged epitopes, such as the ones found on the surface of HBP. The heparan sulphates and similar proteoglycans with GAG side chains may therefore be seen as a proper receptor or co-receptor for HBP.

HBP may also exploit its dipolar nature by activating the contact phase system. The contact phase system consists of HMWK and three other proteins which are closely

bound together on the cell surface. HMWK is a large protein consisting of 6 domains, of which one (domain 4) contains the Bradykinin sequence. An electrostatic binding from a positively charged histidin-rich area in domain 5 of HMWK to negatively charged heparan sulphate and chondroitin sulphate proteoglycans contribute significantly to the binding of HMWK to cell surfaces. The activation of the contact phase system requires that the individual components (HMWK, fXII and prekallikrein) are brought in close contact to each other and probably also that certain conformational changes are induced. Heparin-binding protein (HBP) has been shown to play a pivotal role in activating the contact phase system, and to be capable of highly effectively displacing HMWK from GAG in an in vitro model (Renne, T. 1999). This occurs most likely by formation of two electrostatic bindings, one between the negatively charged GAG on the cell surface and HBP's strongly positively charged surface area, and another between the positively charged domain 5 of HMWK and HBP's negatively charged surface area.

Further, in addition to the above-mentioned highly charged binding sites HBP also carries other putative binding sites, such as binding sites for the Lipid A part in LPS and for interaction with and activation of Protein Kinase C (PKC).

Antibodies directed to different epitopes on HBP are of considerable pharmaceutical interest as drug candidates for the prevention and/or the treatment of infections, local and systemic inflammatory disorders, asthma, systemic inflammatory response syndrome (SIRS), degenerative diseases (Alzheimer's disease), pain and other serious diseases and disorders. Functional antibodies against HBP by binding to different epitopes comprised by the binding sites to putative HBP receptors, may modulate the functioning HBP in disease, improving or impairing the HBP capabilities as a mediator of the inflammatory response.

The invention features two groups of anti-HBP functional antibodies:

One group of antibodies is the antibodies that have agoinistic capacities to HBP functions. These antibodies according to the invention are capable of recognition of en epitope(s) located within a fragment of HBP comprising amino acid residues 1 to 19 or 45 to 225 (according to SEQ ID NO: 1) concerns by the invention as powerful

stimulators of different HBP activities involved in mediation of the inflammatory response. These antibodies may according to the invention serve as

(i) clustering agents increasing a local concentration of HBP at the sites of inflammation,

(ii) enhancers of the biological activity of 20-44 fragment of HBP, which has been shown to be of major importance for example for bactericidal function of the protein or in activation of monocytes;

(iii) co-factors that favors the interaction between HBP and a HBP receptor, when the binding of an antibody to HBP leads to a conformational chance in the structure of the protein that favors the interaction.

Another group of antibodies of interest is a group consisting of anti-HBP antibodies having antagonistic capacities to HBP function. These antibodies according to the invention are capable to recognize an epitope(s) within the sequence of HBP comprising amino acid residues 20-44 (SEQ ID NO: 1). Binding to such epitopes according to invention may lead to inhibiting the functions of HBP, such as for example monocyte activation and releasing the mediators of inflammation e. g. IL-6.

Medicament

It is an important objective of the present invention to use the antibodies, functionally active fragments or variants of said antibodies for the manufacture of a medicament for prevention and/or treatment of Gram positive and/or Gram negative infections, sepsis, severe sepsis, septic shock and/or disseminated intravascular coagulation, and/or for modulation of inflammatory response, and/or prevention of cell apoptosis.

In one embodiment the invention relates to the manufacture of a medicament which is capable of being used for prevention and/or treatment of Gram positive bacterial infection caused by Bacillaceae, Micrococcaceae, Mycobacteriaceae, Peptococcaceae and/or a Gram negative bacterial infection caused by Acetobacteriaceae, Alcaligenaceae, Bacteroidaceae, Chromatiaceae, Enterobacteriaceae, Legionellaceae, Neisseriaceae, Nitrobacteriaceae, Pseudomonadaceae, Rhizobiaceae, Rickettsiaceae, Spirochaetaceae, Vibrionaceae, Brucella, Chromobacterium.

In a preferred embodiment for prevention and/or treatment the infection by *Neisserie meningitidis* (meningococcus) and/or *Pneumococcus pneumonae* (pneumococcus).

In another embodiment the invention relates to the manufacture of a medicament which may be used for prevention and/or treatment of sepsis, severe sepsis, septic shock and disseminated intravascular coagulation.

It is an important objective of the invention to use the antibodies for the manufacture of a medicament for stimulation of an inflammatory response, in a preferred embodiment, the inflammatory response to bacterial infection.

Another important objective of the invention is to use the antibodies for the manufacture of a medicament for inhibition of an inflammatory response. Examples of inflammatory responses, which may be harmful for an individual and therefore are advantageously being suppressed include but are not limited by conditions associated with extensive trauma, or chronic inflammation, such as for example type IV delayed hypersensitivity, associated for example with infection by *Tubercle bacilli*, or systematic inflammatory response syndrome, or multiple organ failure, or rheumatoid arthritis.

Specifically, antibody F19A5B1, which is capable of binding to an epitope comprising one or more amino acid residues of a sequence comprising amino acid residues 1 to 19 or 45 to 225 of the sequence set forth in SEQ ID NO: 1 and thereby stimulating at least one inflammatory response associated with one or more biological activities of hHBP or a hHBP fragment comprising amino acids 20-44 according to the sequence identified as SEQ ID NO: 1, preferably stimulating the production of IL-6, may be used for the manufacture of a medicament for stimulation of an inflammatory response, in a preferred embodiment, the inflammatory response to bacterial infection. Accordingly, antibody F19A5B4, which is capable of binding an epitope comprising one or more amino acid residues of a sequence comprising amino acid residues 20 to 44 of the sequence set forth in SEQ ID NO: 1 and thereby inhibiting the inflammatory response associated with one or more biological activities of hHBP or a hHBP fragment comprising amino acids 20-44 according to the sequence identified as SEQ ID NO: 1, preferably inhibiting the production of IL-6, may be used for the manufacture of a medicament for a pathological condition wherein inhibition of

inflammatory response is required. Examples of inflammatory responses, when a medicament comprising the inhibitory antibody may be used include but are not limited by the conditions associated with extensive trauma, or chronic inflammation, such as for example type IV delayed hypersensitivity, associated for example with infection by *Tubercle bacilli*, or systematic inflammatory response syndrome, or multiple organ failure, or rheumatoid arthritis.

In an additional embodiment the invention intend to use the above described antibody for stimulating or inhibiting the anti-apoptotic activity of hHBP. Such antibodies may be used for the manufacture of a medicament for treatment of a disease or pathological conditions associated with massive cell loss due to apoptosis. Examples of such a disease include but not limited by degenerative diseases the central and peripheral nervous system, such as postoperative nerve damage, traumatic nerve damage, e.g. resulting from spinal cord injury, impaired myelination of nerve fibers, postischaemic damage, e.g. resulting from a stroke, multiinfarct dementia, multiple sclerosis, nerve degeneration associated with diabetes mellitus, neuromuscular degeneration, schizophrenia, Alzheimer's disease, Parkinson's disease, or Huntington's disease, degenerative conditions of the gonads, of the pancreas, such as diabetes mellitus type I and II, of the kidney, such as nephrosis, or cancer.

By the term "apoptosis" in the present content is meant a programmed cell death due to activation an internal death program.

In the pharmaceutical composition of a medicament according to the invention, the antibodies may be formulated by any of the established methods of formulating pharmaceutical compositions, e.g. as described in Remington's Pharmaceutical Sciences, 1985. The composition may typically be in a form suited for local or systemic injection or infusion and may, as such, be formulated with sterile water or an isotonic saline or glucose solution. The compositions may be sterilised by conventional sterilisation techniques, which are well known in the art. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilised, the lyophilised preparation being combined with the sterile aqueous solution prior to administration. The composition may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents and the like, for instance sodium acetate, sodium

lactate, sodium chloride, potassium chloride, calcium chloride, etc. The concentration of peptides may vary widely, i.e. from less than about 0.5%, such as from 1%, to as much as 15-20% by weight. A unit dosage of the composition may typically contain from about 10 mg to about 1 g of a peptide.

The antibodies may be administered topically or by injection. Dosages will be prescribed by the physician according to the particular condition and the particular individual to be treated. Dosages and frequency is carefully adapted and adjusted according to parameters determined by the physician in charge. A preferred administration route may be e.g. subcutaneous injections. Subcutaneous, intravenous, intramuscular, intratracheal, intravesical, intratechal or intraperitoneal injections of anti-HBP antibodies may be given per 24 hours in the range of from 0.1-100 mg, especially 0.1-20 mg, in particular 0.1-10 mg per kg body weight. The dose may be given 1-4 times per 24 hours or administered continuously through a catheter.

Compositions of a medicament used in the present invention comprising bioactive anti-HBP antibodies described above may additionally be supplemented by antibiotics, wherein said antibiotics are routinely prescribed antibiotics by the physician according to the particular condition and the particular individual to be treated. In a preferred embodiment the supplemented antibiotics are selected from but not limited by the group of beta-lactam antibiotics, comprising penicillins and cephalosporins. A medicament comprising an anti-HBP antibody may still additionally be supplemented by an pro-inflammatory drug, or an anti-inflammatory drug, wherein said drugs are prescribed by the physician according to the particular condition and the particular individual to be treated. The supplementary pro-inflammatory drugs may for example be selected from the group comprising CSF (colony stimulating factor) drugs. The supplementary anti-inflammatory drugs may for example be selected from the group comprising antibiotics, steroids, cytostatics, or antiviral drugs.

Experimentals

Example 1: Production of monoclonal antibodies

Methods

Production of monoclonal antibodies

A panel of monoclonal antibodies against pHBP were produced according to method of Kohler G. and Milstein C (Nature 1975;256:495-497). The antibodies were purified using ImmunoPure^{R} IgG (Protein A) Purification Kit (PIERCE) and tested in the whole blood assay as described below.

Purified antibodies have been screened for a capability to inhibit or stimulate production of IL-6 in whole human blood in the presence or absence of h20-44 peptide alone or in the presence of h20-44 peptide and PGN or PCW from Staphilococcus aureus.

Whole blood screening assay

Human whole blood (WB) samples contain besides red cells, platelets and plasma the white blood cells including the neutrophils and monocytes. Neutrophils and monocytes have receptors for bacterial products such as LPS, PGN and LTA. The bacterial products react directly or via specific binding proteins to receptors on the monocytes thereby stimulating them to secrete and release inflammatory cytokines such as IL-1, IL-6, and TNF-α. HBP and HBP fragments, specifically the fragments comprising 20-44 aa according to SEQ ID NO: 1 or 2 do not influence the production of cytokines when they present in the blood, however they can significantly amplify cytokine synthesis and secretion induced by bacterial products. In the assay the amplification of 160 µM HBP per ml WB typically leads to at least three-fold amplification of the cytokine secretion.

In the screening assay LPS from the E. coli was used to stimulate the monocytes in WB (anti-coagulated by use of citrate) to secrete IL-6 and activity of the antibodies was evaluated in relation to the amount of IL-6 in plasma subsequently separated from WB. The activity was related to the capability to:

5. increase IL-6 secretion in absence of bacterial products
6. increase IL-6 secretion in presence of bacterial products
7. decrease IL-6 secretion in presence of bacterial products
8. inhibit amplification of IL-6 secretion induced by intact HBP

Reagents and methods

All operations must be carried out in LAF cabinet by observance of stringent aseptic techniques. All test tubes, pipette tips etc. must be pyrogen-free. Buffers must be prepared by use of sterile, pyrogen water, preferably water for injection. Use 0.1 % pyrogen-free BSA/PBS for all dilutions.

Add 20 µl of the antibody (in concentrations from 25 to 2500 µg/ml) to 100 µl freshly drawn (less than 4 hours old) citrate whole blood from a healthy human volunteer. Add 20 µl bacterial component (LPS, LTA or PGN) in concentrations from 5 to 5000 ng/ml, preferably 50 to 500 ng/ml. Mix well and incubate for 16-18 hours in an atmosphere of 5 % carbon dioxide and at least 95 % relative humidity. At the end of the incubation add at least 5 volumes (700 µl) 0.1 % BSA/PBS. Mix well. Centrifuge 10 min. at 10.000 g. Aspirate 500 µl supernatant. Determine the level of IL-6 by a specific human immune assay for human IL-6 with sensitivity of at least 3 pg/ml, e.g. Human IL-6 Kit from RnD Systems (cat. no. D 6050).

Negative controls: 100 µl WB plus 40 µl 0.1 % BSA/PBS. Positive control: 100 µl WB plus 20 µl LPS (same concentration as used for testing the peptide) and 20 µl 0.1 % BSA/PBS.

Results

Table 1

Blood µl	F19A5B1 mg/ml	F19A5B4 µg/ml	pHBP mg/ml	0.1%BSA µl	PCW µg/ml	IL-6 pg/ml
100				60		10.4
100				60		13.0
100	168			40		3213.6
100	168			40		3857.1
100		168		40		10.4
100		168		40		14.3
100	168		25	20	90	5255.9
100	168		25		90	4755.4
100		168	25	20	90	6.5
100		168		20	90	1.4

From table 1 it appears that the monoclonal antibody produced by clone F19A5B1 (fusion No19 well A5 sub-cloned and well B1 selected) enhanced the IL-6 secretion when added alone, and also stimulated the IL-6 production when added together with PCW and pHBP in the whole blood assay described above. From the table it also appears that the monoclonal antibody F19A5B4, obtained from well 4 in the same sub-cloning of F19A5, had the opposite effect resulting in inhibition of the IL-6 production when added either alone of in the presence of PCW and pHBP.

Table2

Blood ml	**hHBP 20-44 peptide µg/ml**	**F19A5B4 mg/ml**	**0.1%BSA**	**PGN mg/ml**	**IL-6 pg/ml**
100	360		40		510.5
100	360		40		366.6
100	180		40		122.7
100	180		40		142.7
100	90		40		60.7
100	90		40		51.9
100			40	250	15331.1
100			40	250	13856.5
100	360		20	250	27639.4
100	360		20	250	23178.0
100	180		20	250	19289.1
100	180		20	250	22415-3
100	90		20	250	14668.9
100	90		20	250	8109.7
100	45		20	250	10619.0
100	45		20	250	9783.6
100	360	168	20		317.4
100	360	168	20		255.7
100	180	168	20		80.7
100	180	168	20		70.6

100	90	168	20		56.8
100	90	168	20		60.7
100	45	168	20		17.3
100	45	168	20		16.4
100	360	168		250	3638.7
100	360	168		250	2615.7
100	180	168		250	730.1
100	180	168		250	1006.4
100	90	168		250	337.7
100	90	168		250	145.0
100	45	168		250	60.1

From table 2 it appears that human 20-44 peptide acetylated in the N terminal and amidated in the C terminal respectively enhanced the IL-6 secretion when added alone and also enhanced the IL-6 secretion when added together with PGN. However, the monoclonal antibody F19A5B4 inhibits these effects of the peptide.

Conclusion

Highly specific monoclonal and also polyclonal antibodies raised against HBP or peptides thereof can be produced and selected on their capability to stimulate the immune response (measured as IL-6 secretion but not restricted to this cytokine as a number of other cytokines such as TNF-alpha, IL-1, IL-8 can also be measured in the same time). Thus, compounds comprising such antibodies would potentiate the immune response induces by bacterial products

Highly specific monoclonal and also polyclonal antibodies raised against HBP or peptide fragmetns thereof can be produced and selected for their capability to inhibit the immune response (measured as IL-6 secretion but not restricted to this cytokine as a number of other cytokines such as TNT-alpha, IL-1, IL-6 can be measured in the same time). Such antibodies will inhibit the immune response induced by bacterial products and/or the immune response induces by endogenous HBP or peptide fragments thereof added exogenously, specifically the response induced by the fragment of human HBP consisting amino acids 20-44 according to the sequence identified as SEQ ID NO: 1, wherein the C-terminus of said fragment is acetylated and the N-terminus is amidated. Thus, a compound comprising such antibodies

would inhibit the amplification of the immune response induces by the endogenous HBP or HBP peptide fragments added exogenously in the presence of bacterial lipopolysaccarides in the blood.

Example 2. Reduction of toxic shock syndrome lung damage by anti-HBP antibody

Background

Streptococcus pyogenes is a major human bacterial pathogen, which frequently causes a severe illness known as hyperacute toxic shock syndrome (TSS). TSS is characterized by extensive plasma leakage, circulatory shock, and fibrin deposits into the lungs causing respiratory failure with high mortality rate. During an infection *Streptococcus pyogenes* expresses and releases substantial amounts of the M protein, which is an exotoxin responsible for the virulence of this bacteria and the major pathogenic factor in TSS.

Mouse model of severe toxic shock syndrome

Dr. Heiko Herwald and colleagues at Lund University, Sweden, have developed a model for provocation of TSS in mice. In this model, M protein is injected intravenously (i.v.) into mice. Within 30 minutes the mice develop signs of severe shock. The mice are sacrificed after 30 minutes and the lungs are examined by scanning electron microscopy, which show evidence of severe destruction of the normal alveolar structure and extensive fibrin deposits, which can be seen as evidence of extensive plasma leakage. HBP, a central pro- inflammatory mediator, plays a significant pathogenic role in the toxic shock syndrome caused by the Streptococcus pyogenes M protein. Anti-human HBP antibody significantly reduces lung damage. Mice have an HBP-like molecule, which is capable to reacts with certain antibodies raised against human HBP. In vitro studies have shown that such antibodies significantly inhibit the inflammatory reactions caused by the M protein. It was therefore evaluated if such antibodies could also prevent or reduce the lung damage caused by i.v. injection of the M protein in mice. Adult mice were divided into three groups, 3 mice in each group. The first group served as control and received only PBS. The second group received 15 µg M protein intravenously and the third group received 15 µg M protein plus 100 microgram of a polyclonal rabbit anti- human HBP anti-

body previously shown to cross-react with the putative mouse HBP molecule. Thirty minutes after the injection the mice were sacrificed and the lungs removed.

Results

The lungs were examined by scanning electron microscopy. The control group as expected showed normal lung structure. In the group given the M protein, severe lung damage was observed: the alveolar structure was destroyed, the remaining walls were thickened and the alveoli were filled up with deposits. In the group given M protein and anti-HBP antibody it appeared that between 2/3 and 3/4 of the alveoli and normal alveolar structure were preserved, indicating a dramatic reduction in the lung damage. Careful examination of all the electron microscopy pictures taken revealed the same observations in all three mice within each group.

Discussion and conclusion

Human HBP has been shown to be responsible for neutrophil evoked vascular leakage in an *in vitro* model (Gautam et al. Nature Medicine 2001;7:1-5). Anti-HBP antibodies might be an interesting approach to development of a new class of anti- inflammatory drugs. The results presented provide the first *in vivo* evidence that anti-HBP antibodies may actually be therapeutically useful in prevention of severe inflammatory lung damage.